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Optimized method for analysis of ethanolamines, hydrolysis products of nitrogen mustards, from urine samples using LC-MS/MS

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ABSTRACT

Highly polar ethanolamines (EAs), excreted in urine, are hydrolysis products of nitrogen mustards (NMs), which are prohibited by the Chemical Weapons Convention (CWC). The methods established for biological matrices are essential for verification analysis of the CWC related chemicals. This paper describes a new liquid chromatography-tandem mass spectrometry (LC-MS/MS) method developed for qualitative and quantitative analysis of EAs, N-ethyldiethanolamine (EDEA), N-methyldiethanolamine (MDEA) and triethanolamine (TEAOH) from urine samples. After optimization of sample preparation and chromatographic conditions, the method was fully validated. Silica solid-phase extraction (SPE) cartridges and a porous graphite carbon (PGC) column were selected for validation studies. The method is linear from 5 to 500, 0.5 to 250, and 0.5 to 500 ng/mL for TEAOH, EDEA, and MDEA, respectively. It is also precise and accurate. A minimum sample amount of 0.5 mL urine was used. The limit of quantification using this approach was 0.4, 5.5, and 6.3 ng/mL for MDEA, EDEA and TEAOH, respectively. The combination of the PGC column and high pH eluents in analysis retained and separated the studied EAs. Retention times were 2.11, 2.56 and 2.98 min for MDEA, EDEA and TEAOH, respectively. The method is applicable for verification analysis of the CWC.

1. Introduction

Nitrogen mustards (NMs) are alkylating agents and classified as toxic chemicals in Schedule 1 in the Chemical Weapons Convention (CWC) [1]. NMs include bis(2-chloroethyl)ethylamine (HN1), bis(2-chloroethyl)methylamine (HN2), and tris(2-chloroethyl)amine (HN3). The use of NMs as chemical weapons (CWs) has not been documented, even though they could be used in chemical terrorism. This might be because of their short lifetime as they become unstable after long-term storage of one to three months [2]. In view of the low persistency in the environment, biomedical would be an important source of evidence for the investigation of alleged use of these CWs. Thus, the development of methods for detecting CWs in biomedical samples would provide qualitative and quantitative evidence of CWAs exposure. The Organization for the Prohibition of Chemical Weapons (OPCW), CWC implementing agency, will send authentic samples collected by OPCW inspectors to designated laboratories (DLs) to examine the use of any prohibited chemicals. There is a need to develop highly selective and sensitive methods to detect these chemicals in different matrices with a limited sample at low concentration. A method that can be used in a great variety of situations is preferable.

The NMs decompose to ethanolamines (EAs) via hydrolysis between carbon atoms and a leaving group (Cl) [3]. N-ethyldiethanolamine (EDEA), N-methyldiethanolamine (MDEA) and triethanolamine (TEAOH) are hydrolysis products of HN1, HN2 and HN3, respectively [4]. Their molecule structures and their pKa values are given in Fig. 1. TEAOH, which is widely used in industrial and domestic cleaning products and cosmetics, can be endogenous in high concentrations in human urine [5]. Consequently, other biomarkers are needed to confirm exposure to HN3.

Several papers have reported a variety of analytical techniques for EA analysis such as capillary electrophoresis (CE) [6], gas chromatography-electron impact/mass spectrometry (GC-EI/MS) [7–11], liquid chromatography-tandem mass spectrometry (LC-MS/MS) [12–17], and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOFMS) [18]. Tandem mass spectrometry offers better sensitivity and selectivity than single-stage MS. Detection limits ranged from parts per million (ppm) to as low as parts per billion (ppb).

Various sample preparation involving solid-phase extraction (SPE)

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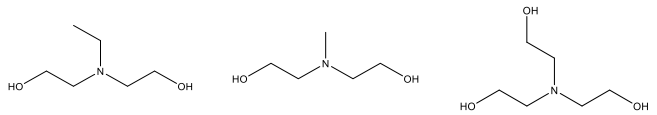


Fig. 1. Chemical structures and pKa values of EAs (accessed from Pubchem).

[7,12,13,17,19–22], derivatisation [9–11,14,15], and solvent extraction [17] are used for detection of EAs in different matrices like urine [10,11,19,21,22], water [7,11,14,17], decontamination solution [17], plasma [7,23], and serum [23]. Different types of SPE sorbents have been applied, namely strong cation exchange (SCX) [7,22,24], and non-polar (C18) [13] sorbent depending on the studied matrices for EA analysis. NMs themselves have also been extracted using a silica SPE cartridge and acetone as an extraction solvent [20].

Several derivatizing agents have been used for GC–MS analysis of EAs such as 1-(heptafluorobutyryl)imidazole for aqueous samples [14], *tert*-butyldimethylsilyl (TBDMS) for water, urine and blood samples [11]. Derivatization steps sometimes contribute to low recoveries, and they are time-consuming since concentration to dryness as part of sample treatment is required. It can also be a significant error source when extraneous materials react with the derivatizing agent to produce a complex background [9]. To overcome these issues, LC-MS has been used to analyse EAs during recent years [25].

Most of the research on bioanalytical methods for NMs and EAs have been done from the blood (whole, plasma, serum) and urine samples. These are the most available samples that accumulate a high proportion of the dose in surviving casualties [24]. Excretion via urine is a main route of elimination. However, it has the disadvantage that the major portion (~90%) of the metabolites formed are excreted within the first 72 h after exposure. Numerous methods for the detection of EAs in urine have been developed [10,19,21,22]. Most of the published methods for urine samples use SCX sorbents and separate the EAs chromatographically using hydrophilic interaction chromatography (HILIC) and LC-MS/MS [19,22]. Another method uses ACN for extraction of water samples and a mixed-mode ultra high performance liquid chromatography (UHPLC) column with embedded ion-pairing groups (exhibiting both hydrophobic and cation exchange retention mechanisms) for chromatographic separation [17]. There are also established methods to detect the exposure of NMs in blood matrices [13,23,26]. These methods via DNA and protein adducts are preferable because NM adducts to proteins such as albumin and haemoglobin are ideal as they are stable for several weeks to months [23].

This paper focuses on NM hydrolysis products in urine samples. Also, this study aimed to develop an analytical method for extracting these highly polar compounds from a urine sample while at the same time simplifying the sample preparation procedure and reducing the sample volume in use. Several SPE sorbents and LC columns were tested, and a method using silica SPE and porous graphitic carbon (PGC) was optimized and validated for analysis of EAs from urine samples. The use of silica SPE sorbent combined with PGC column was highly effective for retaining EAs and reduced urine sample background. To our knowledge, this is the first-time a combination of high pH mobile phase and a PGC column was used to separate highly polar analytes.

2. Material and methods

2.1. Materials and reagents

Reference chemicals of EDEA and MDEA were synthesized at VERIFIN, and TEAOH was obtained from Sigma Aldrich (St. Louis, MO, USA). NMR was used to determine the concentrations of stock solutions of reference chemicals, and the purity of the stocks ranged from 69% to 88%. D_{15}-TEAOH was obtained from CDN Isotopes (Essex, UK). This compound was found to be D_{12}-TEAOH after purity check by NMR and LC-MS/MS. The stock solutions were prepared by dissolving the analytes in ACN and stored at 4 °C. The working solution was prepared for the appropriate concentration by diluting with purified water before use.

A few types of SPE cartridges and LC columns were tested in this study as shown in Table 1. Methanol (MeOH) and ACN were obtained from Honeywell Fluka (Loughborough, LE, UK) and JT Baker (Phillipsburg, NJ, USA), respectively. Other chemicals and reagents such as ammonium acetate (NH_4OAc) and ammonium formate (NH_4HCO_2) were purchased from Sigma Aldrich (St. Louis, MO, USA), formic acid for LC-MS (98–100%) from Merck (Darmstadt, Germany) and ammonium hydroxide solution (NH_4OH, ≥25% in H_2O), eluent additive for LC-MS grade from Honeywell Fluka (Seelze, Germany). All chemicals and reagents were of analytical grades or higher. The water used in this study was purified using a Direct-Q3 UV system (Millipore, Germany). A pooled human urine was provided from six healthy volunteers (three males and three females).

2.2. LC-MS/MS

LC was performed by Waters Acquity UPLC i-Class from Waters Corporation (Milford, MA, USA). This UHPLC system was coupled to a Waters Xevo TQD triple quadrupole mass spectrometer from Waters Corporation (Milford, MA, USA). A PGC column was used as the stationary phase at 40 °C. Solvent A (5 mM NH_4OAc, pH 11.0) and solvent B (5 mM NH_4OAc in MeOH, pH 11.0) were used as mobile phase in the validation studies. Masslynx (version 4.1) was applied for utilizing data acquisition and quantitative analysis.

The optimized chromatographic conditions consisted of initial conditions of 1% B gradually increased to 100% B in 5 min. It was then held for 1 min before the B ratio was decreased to 1% B in 0.3 min, and the column was equilibrated for 1.2 min at 1% B. The flow rate was 0.4 mL/min, and the injection volume was 5 μL.

The acquisition was performed in multiple reaction monitoring (MRM) mode after positive electrospray ionization (ESI) with nitrogen (N_2) as the spray gas and argon (Ar_2) as the collision gas. The instrument parameters were set as follows: capillary voltage 500 V, cone voltage 32 V, source temperature 150 °C, desolvation temperature 500 °C, cone gas flow 160 L/hr, desolvation gas flow 1000 L/hr and collision gas flow 150 mL/min.

2.3. Sample preparation

A mixed standard solution of EAs and internal standard (ISTD, D_{12}-TEAOH) were prepared in purified water. An appropriate amount of mixed standard solution of EAs was spiked into 0.5 mL of blank urine samples to prepare a calibration curve ranging from 5 to 500 ng/mL for TEAOH, 5 to 250 ng/mL for EDEA, 0.5 to 500 ng/mL for MDEA and QC sample (25 ng/mL). 50 μL of ISTD (250 ng/mL) was added to all samples to give a final concentration of 25 ng/mL. The spiked samples (calibration standards and QC samples) were mixed for 10 s on a vortex mixer and left at room temperature for at least 1 h before SPE clean up.

Table 1
SPE cartridges and LC columns tested in this study.

SPE cartridges	LC columns
Bond Elut Jr strong cation exchange (SCX), 500 mg/ 6 mL), 40 μm, Agilent (Wilmington, DE, USA)	ACQUITY UPLC BEH C18 column, 2.1 × 100 mm, 1.7 μm, Waters (Milford, MA, USA)
Strata® SI-1 Silica (55 μm, 70 A), 500 mg/ 6 mL), Phenomenex (Torrance, CA, USA)	BEH Amide column, 2.1 × 100 mm, 1.7 μm, Waters (Milford, MA, USA)
HyperSep™ Hypercarb™ SPE Cartridges, 200 mg/3mL, 30 μm, Thermo Scientific (San Jose, USA)	Porous graphitic carbon (PGC) Hypercarb™, 2.1 × 100 mm, 3.0 μm, Thermo Scientific™ (San Jose, USA)

SPE protocols such as sample dilution, conditioning, washing, and eluting solvents were established for this study. The pH of the urine sample was measured before analysis. It was then basified to approximately pH 11.0 using 0.2 M NH_4OH before following the SPE protocols mentioned below. For sample dilution, 5 mL of water was added into 0.5 mL of a urine sample and vortexed for 10 s before loaded into the SPE cartridge. ACN (2 × 3 mL) was used for pre-conditioning the silica SPE cartridges. Then, a total of 5.5 mL of a diluted urine sample was loaded on the pre-conditioned cartridge. The cartridge was washed with ACN (2 × 3 mL). 75% methanol (MeOH) containing 1% formic acid (2 × 3 mL) was used for eluting the EAs from the SPE cartridge. The eluate was concentrated to dryness under a gentle stream of nitrogen in a TurboVap® LV II (Caliper Life Sciences, Hopkinton, MA, USA) at 45 °C and 7.5 psi. Finally, the residue was dissolved in 400 μL of 5 mM NH_4OAc, pH 11.0 (mobile phase A) before LC-MS/MS analysis.

3. Results and discussion

3.1. Optimization of LC-MS/MS parameters

Initially, when the reversed-phase C18 column was tested, the EAs were not retained well on the column because of their hydrophilic nature. Therefore, a BEH Amide (HILIC column) was tested to separate the three hydrolysis products. The HILIC column showed high intensity and symmetry of peaks for all analytes, but the analytes were not well separated. Despite trying several gradient profiles and buffer compositions, sufficient separation of analytes was not achieved using the HILIC column. Carryover was also observed mainly for MDEA transitions. Various washing solvents for injector were also tested; however, it did not resolve the carryover problem.

Eventually, a PGC column was selected because of its ability to retain very polar compounds and separate closely related compounds. This column is also stable throughout the entire pH range of 1 to 14, and it is proven to withstand aggressive mobile phases. It also worked well to resolve EAs. The retention of EAs in the PGC column is based on the mechanism of charge induced interactions of these highly polar analytes with the polarizable surface of graphite and delocalized electrons on the graphite surface. This graphite behaves both as an electron donor and electron acceptor.

There were also no significant differences for changing the column temperature from 40 °C to 60 °C (data not shown).

The PGC chromatographic system was first tested under acidic conditions (0.1% formic acid in 5 mM NH_4HCO_2/ACN). When the studied EAs were analysed under this condition, the peaks eluted early with suboptimal peak shape and analyte resolution (data not shown). Next, a basic eluent system (5 mM NH_4OAc in water/MeOH) was tested (pH 8.4). The separation was sufficient and showed better peak shapes. Both eluent systems were evaluated using isocratic and gradient modes. For

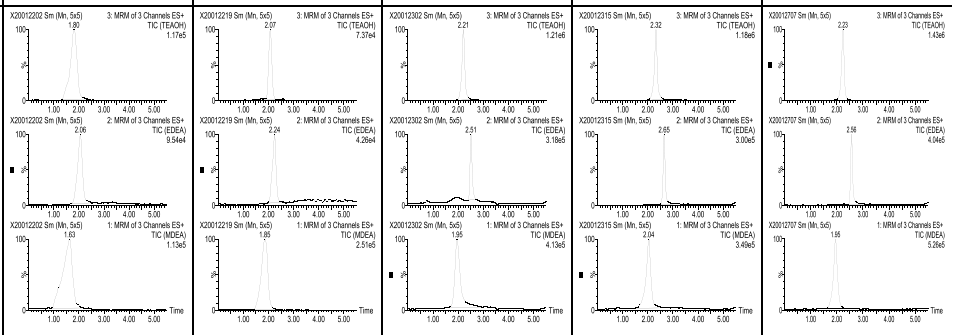
pH		pH 5.5	pH 7.0	pH 9.5	pH 11.0	pH 12.0
Factors						
TEAOH	RT (min)	1.80	2.07	2.21	2.32	2.23
	Absolute intensity (%)	1.17 exp5	7.37 exp4	1.21 exp6	1.18 exp6	1.43 exp6
	Peak width (min)	0.8	0.3	0.5	0.5	0.4
	Symmetry factor	0.9	0.9	1.6	0.8	1.0
EDEA	RT (min)	2.06	2.24	2.51	2.65	2.56
	Absolute intensity (%)	9.54 exp4	4.26 exp4	3.18 exp5	3.00 exp5	4.04 exp5
	Peak width (min)	0.6	0.4	0.3	0.3	0.3
	Symmetry factor	1.3	0.9	1.4	1.2	1.3
MDEA	RT (min)	1.63	1.85	1.95	2.04	1.95
	Absolute intensity (%)	1.13 exp5	2.51 exp5	4.13 exp5	3.49 exp5	5.26 exp5
	Peak width (min)	1.0	0.7	0.6	0.6	0.6
	Symmetry factor	0.8	1.1	1.2	1.0	0.9

Fig. 2. A comparison of different pH of LC eluents (5 mM of NH_4OAc in water/MeOH) for 10 ng/mL of reference standard EAs.

isocratic mode, the peaks only separated when a higher aqueous composition was used; however, the isocratic method showed broader peak shapes. Later, gradient mode was applied to optimize the retention and the peaks shape of EAs. Fig. 2 shows the tested eluent pH conditions, ranging from 5.5 to 12.0, and evaluated using gradient mode.

For the validation studies, an eluent system with a higher pH (pH 11.0) was chosen instead of pH 9.5 because it showed a lower background, especially for EDEA transitions. The basic eluent at pH 12.0 was not selected because it is too close to the UHPLC system's maximum pH operating capacity (pH 12.5). Eluents with a higher pH (pH 11) likely gave the best chromatographic separation because, under these conditions, the EAs exist as free bases.

The LC-MS/MS method was optimized using reference standards of the studied chemicals at a 10 ng/mL concentration level. Protonated molecules were used as the precursor ions. Collision energies for all product ions after optimization were 15 eV. The mass transitions are presented in Table 2.

3.2. Optimization of sample preparation

3.2.1. Recoveries in different SPE cartridges

Three replicates of EAs for each studied SPE cartridges (silica, PGC and SCX) were used to determine the recoveries for the SPE cartridge extraction studies. The analytical results of extracted samples (n = 3) were compared with corresponding extracts of blanks spiked with the analyte (n = 1) post-extraction. Both extracted and unextracted (post-extraction) were prepared in a blank urine matrix, and the concentration of EAs was 100 ng/mL. The EAs were spiked in a blank urine matrix for the extracted sample replicates before following the SPE protocols mentioned in Section 2.3. For the unextracted QC, the EAs was spiked into eluate (blank urine matrix undergo SPE extraction) before the drying process. The percent recovery was calculated based on Equation S1. The recoveries of extraction methods using these three different SPE cartridges for EAs in urine samples are shown in Fig. 3. Based on the results, this extraction method using silica SPE cartridges was found to offer optimal recoveries for all three EAs. Primarily, SCX SPE cartridges were tested. While the degrees of recovery were acceptable, high matrix interference and increased background were observed for EDEA and MDEA transitions (data not shown), even though the analysis was carried out as described in a previously published method [19]. Sodium (Na) ion or any other intrinsic ions in urine samples may have contributed to the matrix effects, causing ion suppression when insufficiently removed by the SCX cartridges.

Subsequently, normal phase silica SPE cartridges were tested because of their ability to extract polar analytes. According to Recommended Operating Procedures (ROPs) [27], SPE using silica cartridges and ACN extraction is the most common method used to extract CWAs from organic samples. ACN was chosen to extract EAs from urine sample because of its capability to disrupt the H-bond between EAs with the "–OH" group present on the silica surface.

A PGC-based SPE cartridge (HyperSep™ Hypercarb™ SPE Cartridges) was also tested based on the same chemistry used in the PGC LC column. The same conditions and procedures used in Silica SPE cartridge were applied to this PGC SPE cartridge. Unfortunately, the PGC cartridges showed low recovery for the EAs from urine samples. The SPE protocol may need more extensive optimisation for sufficient recovery and clean up of urine samples.

Table 2
Mass Spectrometric parameters.

Analyte	$[M+H]^+$	product ion, *m/z*
EDEA	134	72 (Q), 88 (q), 116
MDEA	120	58 (Q), 45 (q), 102
TEAOH	150	70 (Q), 132 (q), 88
D_{12}-TEAOH	162	58 (Q), 96 (q)

Q quantifier ion and q qualifier ion

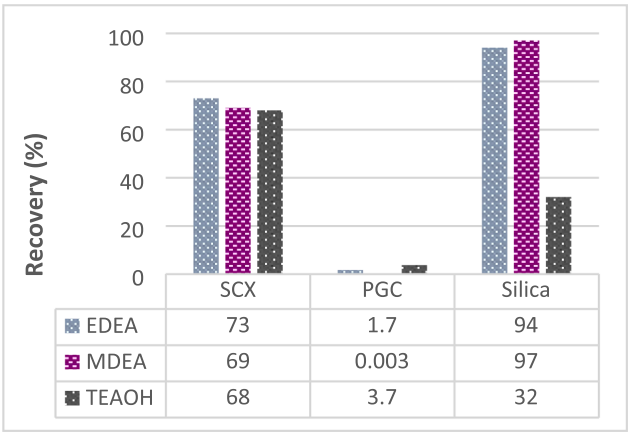


Fig. 3. The recoveries of the extraction methods for three types of SPE cartridges (n = 3). The concentration of EAs in spiked urine was 100 ng/mL. EAs were chromatographically separated by a PGC column using optimized gradient mode in basic LC eluent (5 mM of NH_4OAc in water/MeOH; pH 11).

Extracted ion chromatograms (EIC) of reference chemicals of EAs, blank urine and spiked urine cleaned up by Silica SPE cartridge are given in Fig. 4. The concentration of EAs for both reference chemicals and spiked urine was 25 ng/mL. The final concentration of ISTD in all urine samples was 25 ng/mL. The reference chemicals (A) were prepared by spiking mixed standard solution of EAs and ISTD into eluate before the drying process. The blank urine (B) was prepared by adding 50 µL of ISTD (250 ng/mL) and 50 µL of purified water into a urine sample. For spiked urine (C) and blank urine, the ISTD or mixed standard solution of EAs were spiked before SPE procedures. The reconstituted samples were analysed via LC-MS/MS.

3.2.2. Matrix interferences

There is no contribution for TEAOH signal from the D_{12}-TEAOH. In blank urine, a TEAOH signal was present at five times weaker concentration compared to the 25 ng/ml spiked standard (Fig. 4). Since TEAOH is ubiquitously used in cleaning products and cosmetics, it can typically also be found in human urine without exposure to NMs [5]. There was also interference in EDEA transitions; however, it only appeared at *m/z* 116 transitions (third product ion). This transition is eluted at a different RT than that of the EDEA transition peaks (EDEA RT = 2.92); therefore this transition was omitted for the validation analysis. For MDEA transitions, there was also interference seen from urine background, which eluted at 2.24 mins, 0.2 min after MDEA peaks (MDEA RT = 1.99). This interference was intense at the *m/z* 102 transition, and it was the reason *m/z* 58 and 45 were chosen as quantifier and qualifier ions for this chemical. The integration process should be done precisely and consistently, particularly at a low concentration level. The effect of pH on the urine samples was also studied. Silica SPE cartridge was used in this study. Urine samples were adjusted to lower pH (acidified) using 0.1% formic acid and higher pH (basified) using 0.2 M NH_4OH. The recoveries of EAs increased when urine samples were treated using higher pH, as shown in Supplementary Material (Table S1). The recoveries for basified urine were 32%, 97% and 99.5% for TEAOH, EDEA and MDEA, respectively.

3.2.3. Optimisation of the SPE procedures

Initially, 1 mL of urine was used in method development according to previously published methods [19,22]. However, when the volume was decreased by half (0.5 mL), it showed the same peak intensity as in higher volume (1 mL). A reduced sample volume for the final sample preparation is a significant improvement. ACN and water were also tested as a dilution solvent to dilute urine samples. Water was chosen

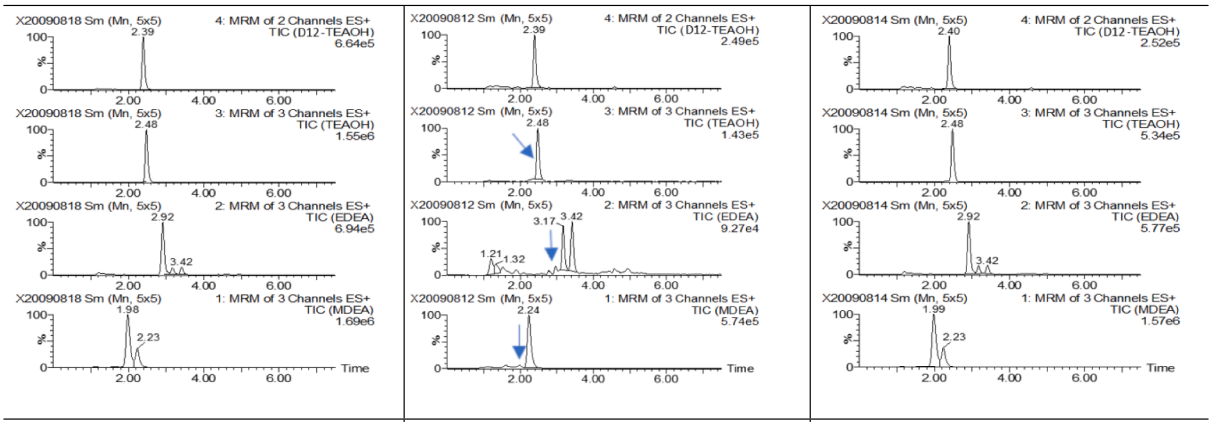


Fig. 4. EIC of EAs in urine extracted by Silica SPE cartridge. The concentration of EAs for reference chemicals (A) and spiked urine (C) was 25 ng/mL. Only ISTD was spiked in blank urine (B). The analytes were chromatographically separated by PGC column using optimized gradient mode in basic LC eluent (5 mM of NH_4OAc in water/MeOH; pH 11).

because it gave higher recoveries for EDEA and MDEA from the urine sample. The details of the study of the dilution solvent and corresponding recoveries are stated in Supplementary Material (Table S2).

ACN was also evaluated to pre-conditioning of the silica SPE sorbent instead of methanol due to its nucleophilic nature. A higher ACN concentration is required to allow interaction of the compounds with the silica sorbent [27]. ACN was also used as a washing solvent. In this study, ACN effectively improved EAs recoveries from the urine sample, as demonstrated in Supplementary Material (Table S3).

Next, MeOH was chosen as an elution solvent because it was miscible with the loading and washing solvents. It also has a higher elution capacity than ACN to elute EAs from the silica SPE cartridges. Four different MeOH mixtures, namely 50%, 75%, 1% formic acid in 50% MeOH and 1% formic acid in 75% MeOH were prepared and tested to optimize the elution solvent. The mixture of 1% formic acid in 75% MeOH gave higher recoveries compared to others. The addition of formic acid substantially enhanced the EAs recoveries. The more organic solvent in the elution mixture also shortened the drying process. The details of the study of elution solvents and corresponding recoveries are shown in Supplementary Material (Table S4).

3.3. Method validation

This method was validated and included all the performance criteria needed, such as linearity, selectivity, accuracy, sensitivity precision, and recovery. The validation was performed in three days. Three calibration curves were obtained per day (n = 9), and the standards were made by spiking into pooled blank urine that had undergone SPE-treatment. True values for the concentrations of calibration standards and their accuracy (relative standard deviation, RSD) were calculated from nine calibration curves. Single-factor ANOVA (Analysis of Variance) was used to determine the precision (variance within groups and between groups), random error, systematic error (bias), and uncertainty estimation from the calibration standards' true values. The validation results are shown in Table 3.

For linearity studies, nine-point calibration standards ranging from 0.5 to 500 ng/mL were prepared in a blank urine matrix. Calibration curves were established for all analytes by plotting the response against

Table 3
The calculated validation results for EAs.

Chemical	Standard level (ng/mL)	Average concentration (ng/mL)	Average SD	Average RSD (%)	Variance within group (%)	Variance between group (%)	Random error (%)	Systematic error (%)	Combined uncertainty
TEAOH	5	3.6	0.6	16.7	0.5	0.5	13.1	−37.0	±19.5
	10	8.7	1.3	15.3	1.3	0.7	9.6	−14.7	±16.8
	25	24.1	1.7	7.1	2.0	1.0	1.9	−3.7	±9.3
	50	50.3	3.0	5.9	2.6	2.0	4.1	0.6	±6.6
	100	101.8	4.0	4.0	3.0	3.5	3.1	1.8	±4.6
	250	253.4	5.5	2.2	5.1	3.4	1.4	1.3	±3.0
	500	498.0	2.3	0.5	2.1	1.4	0.3	−0.4	±1.1
EDEA	5	4.8	0.3	5.9	0.3	0.2	3.9	−3.1	±6.5
	10	10.0	0.5	4.9	0.5	0.2	3.0	−0.1	±5.3
	25	25.7	0.9	3.5	0.8	0.2	2.5	2.8	±4.0
	50	50.8	2.7	5.4	3.2	1.7	1.3	1.6	±7.2
	100	99.5	2.7	5.4	1.7	0.8	0.5	−0.6	±1.9
	250	232.0	16.9	7.3	16.5	8.0	4.4	−7.7	±12.0
MDEA	0.5	0.6	0.1	15.8	0.1	0.1	10.7	18.3	±24.6
	1	1.0	0.1	10.1	0.1	0.1	6.4	−0.8	±12.8
	5	4.7	0.1	2.3	0.1	0.0	0.9	−6.9	±2.8
	10	9.3	0.4	4.4	0.4	0.1	2.5	−7.9	±4.8
	25	25.2	0.9	3.4	0.6	0.8	2.7	0.9	±3.9
	50	50.7	2.1	4.2	2.4	1.0	1.6	1.4	±5.2
	100	104.1	4.5	4.3	5.2	2.3	1.5	4.0	±5.8
	250	250.9	6.7	2.7	7.1	1.0	1.3	0.4	±2.9
	500	469.6	17.4	3.7	10.2	16.8	3.1	−5.4	±13.5

the expected calibrator concentration. A none weighted linear regression was performed for TEAOH and EDEA, and $1/x^2$ for MDEA. The calibration curve for TEAOH using D_{12}-TEAOH as ISTD with the nine points per level are presented in Fig. 5. An average R^2 for TEAOH from 5 to 500 ng/mL was 0.9999. Even though the entire range of 0.5 to 500 ng/mL was linear for TEAOH, the calibration curves were evaluated from 5 ng/mL as its limit of quantitation (LOQ) was 6.3 ng/mL. For EDEA, the curves were linear from 0.5 to 250 ng/mL and an average $R^2 = 0.9992$. For MDEA, the curves also linear for the entire range from 0.5 to 500 ng/mL with an average $R^2 = 0.9902$. Average calibration curves for EDEA and MDEA are presented in Supplementary Material (Figs. S1 and S2). Quality control samples (25 ng/mL) were analysed after every calibration standard run to establish the LC-MS instrument's performances.

The selectivity of the method was examined during validation with a blank urine matrix and purified water. There are some interferences observed for TEAOH in the blank samples as they present in relatively high amounts in human urine [5] due to its use in industrial and domestic cleaning products and cosmetics. Additionally, TEAOH was observed in the LC eluents when a lower-grade NH_4OH was used. This signal was reduced to lower than the LOQ after using higher grade NH_4OH. The use of D_{12}-TEAOH compensated for the effects of this interference. Two transitions in multiple reaction monitoring (MRM) modes were used for each analyte, as presented in Table 2. For TEAOH, the response was calculated from the standard areas divided by the ISTD areas. The EDEA and MDEA responses were obtained from the ratio between qualifier and quantifier ions. The average of q/Q ratio for EDEA and MDEA was 17% and 36%, respectively. According to the European Union (EU) criteria for the mass spectrometric techniques [28], the maximum permitted tolerance for relative ion intensities for EDEA is ±30%, and MDEA is ±25%. It gives the maximum tolerance of ±5 and ±9 for EDEA and MDEA, respectively. The average retention time for TEAOH was 2.56, with RSD 0.3%. For EDEA and MDEA, the retention time was 2.98 and 2.11 with RSD 0.8% and 0.4%, respectively.

The accuracy was determined for each chemical at all studied concentration levels. We followed the Food and Drug Administration (FDA) bioanalytical method validation recommendations that the values need to be within 15% at nominal concentration and less than 20% at the LOQ [29]. These values also applied to precision studies. For TEAOH, all the RSD values obtained were under 15% except for the lowest concentration level, which it was 16.7%. All RSD values are below than 7.3% for EDEA. For MDEA, all the RSD values were within the FDA recommendations (Table 3).

The precision of the method was assessed using variance within group and between groups. For TEAOH, the variance within groups and between group was under 5.1%. The variances value was also acceptable for EDEA except at the highest concentration level (RSD = 16.5%). The values acquired for MDEA were under the limit except for the variance between group at the highest concentration level. Most of the values obtained follow FDA guidelines.

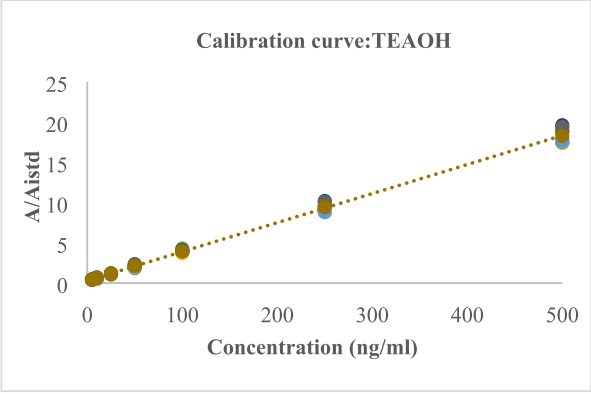


Fig. 5. An average of calibration curves (n = 9) for TEAOH.

The method sensitivity was defined by the limit of detection (LOD) and the LOQ. It was determined from the linear regression from equations (1) and (2), where s is the standard deviation of the signal (y-intercept) and b is the slope of the nine calibration curves prepared in blank urine matrix. Measured LODs were 0.1, 1.7, and 1.9 ng/mL for MDEA, EDEA, and TEAOH, respectively. LOQs for all chemicals are shown in Table 4.

$$\mathrm{LOD} = 3 \times \frac{s}{b} \quad (1)$$

$$\mathrm{LOQ} = 10 \times \frac{s}{b} \quad (2)$$

The within run precision was established using six replicates (n = 6) of spiked samples at four different concentration levels of 5, 25, 100 and 250 ng/mL. The method is precise, and the results obtained are presented in Supplementary Material (Table S5). The highest relative standard deviation (RSD, %) for TEAOH was 6.8% at 5 ng/mL, and the lowest was 2.1% at concentration level 100 ng/mL. For EDEA, the RSD ranged from 2.3% to 8.4% at 25 and 250 ng/mL, respectively. Compared to the other two EAs, MDEA achieved the lowest RSD with just 0.7% at 25 ng/mL. All the RSD obtained during the precision study also complied with the FDA guidelines [29].

The method's recovery was evaluated by spiking three (n = 3) parallel samples at three different concentration levels of 5, 50 and 250 ng/mL. The results are presented in Table 5. The analytical results of extracted samples were compared with corresponding extracts of blanks spiked with the analyte post-extraction [29]. The recovery of TEAOH is low compared to other EAs. At a low concentration level (5 ng/mL), the recovery for TEAOH reached 50%, and it might be contributed from the background effect from urine and LC eluents. For EDEA and MDEA, the recoveries were more than 85% at all levels.

4. Conclusion

This method was fully validated using silica SPE cartridge and PGC column for EAs analysis in the urine sample. It was linear and precise from 5 to 500, 5 to 250, and 0.5 to 500 ng/mL for TEAOH, EDEA, and MDEA, respectively. The method LOQ using this approach was 0.4, 5.5, and 6.3 ng/mL for MDEA, EDEA and TEAOH, respectively. In addition, a lower sample volume was used, and several urine samples can be treated simultaneously using silica SPE cartridges. The optimisation of the SPE procedures also increased the recovery of the highly hydrophilic EAs from urine samples. For LC-MS/MS method, the combination of PGC column and higher pH LC eluents successfully retained EAs and chromatographically separated them. Less than 10 min is required per sample for the instrument analysis. This method can also be applied to water samples.

CRediT authorship contribution statement

Nurhazlina Hamzah: Investigation, Validation, Writing - original draft. **Matti Kjellberg:** Supervision, Writing - review & editing. **Paula Vanninen:** Conceptualization, Writing - review & editing, Supervision, Project administration, Funding acquisition.

Table 4
Method LOD and LOQ for EAs determined from calibration curves (ng/mL).

Chemicals	LOD	LOQ
TEAOH	1.9	6.3
EDEA	1.7	5.5
MDEA	0.1	0.4

Table 5
The recovery results for TEAOH, EDEA and MDEA. Standard deviation (SD), relative standard deviation (RSD) (n = 3).

Chemical	Concentration level (ng/mL)	Average recovery (%)	SD	RSD (%)
TEAOH	5	50	2.2	4.4
	50	34	0.4	1.3
	250	35	0.4	1.3
EDEA	5	86	6.5	7.5
	50	89	2.8	3.1
	250	91	1.3	1.5
MDEA	5	89	1.6	1.8
	50	88	1.2	1.3
	250	95	0.6	0.7

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

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